BIOSYNTHESIS OF HORMONAL AND NEURAL PEPTIDES*, **, †

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MONTRÉAL

Following the discovery of γ-LPH in 1967 and the determination of its complete amino acid sequence (1), we proposed the concept of pro-hormone based on the sequence homology and the structure/bioactivity relationships of this then novel peptide with β-LPH and β-MSH.

We also noticed that the sites of cleavage on the pro-hormone β-LPH were made of pairs of basic amino acid residues. At exactly the same time, Steiner et al. (2), using well designed pulse-chase experiments, revealed that insulin was biosynthesized as a larger molecular weight form. In 1968, when the sequence of pro-insulin became known (3), it was most interesting to note that the sites of cleavage of the peptide connecting both chains of insulin were also made of pairs of basic amino acids. Since then, numerous models have been proposed and subsequently proven (4). The main common feature of all of them is the finding that pairs of basic residues are found at the key positions for posttranslational maturation of the precursors.

We would like to discuss in this article the main methodological approaches to study prohormone models, to present some of the key experiments demonstrating the maturation of pro-ACTH/LPH/endorphin, also called POMC (5), and finally to summarize briefly how the LPH model proposed in 1967 is applicable to all the known models described so far for hormonal and neural peptides.

We wish first to define a number of criteria that must be met before the existence of a biosynthetic precursor for a peptide hormone or secretory protein can be established with certainty. These are based in part on those described previously by Tager et al. (6).

(i) Immunoprecipitation experiments with well-characterized antibodies raised against the hormone must specifically precipitate the higher as well as the lower molecular weight forms of the hormones.

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(ii) "Peptide mapping" of the precursor must demonstrate the existence, within the larger molecular form, of peptides characteristic of the active hormone, together with additional fragments.
(iii) Pulse-labeling and pulse-chase experiments must be carefully conducted to establish the precursor-product relationship between high and low molecular weight forms of the proteins.
(iv) Sequence analysis of the putative precursor must reveal the existence of additional peptide(s) covalently linked to the hormone.

The first two criteria constitute suggestive evidence for the existence of a precursor for a given hormone. The pulse-chase experiments are more indicative while conclusive proof can be obtained only through careful structural analysis of the putative precursor. Pulse-chase experiments and sequencing of the precursor to show an additional fragment covalently linked to the correct sequence for the active hormone are, in our view, the only definitive proof that the larger form is indeed the biosynthetic precursor of the smaller peptide.

Four main approaches derived from the criteria described above have been used to develop the concepts of hormonal or neural peptides or both: 1. immunological characterization of different forms of precursor; 2. classical pulse-chase experiments; 3. direct sequencing of the protein or indirectly with the cDNA.

1. Immunological Characterization

In this approach, immunoprecipitation is used as a preparative tool as well as the main technique for characterizing the biosynthetic peptide. The success of the method depends heavily on the specificity of the antibody. However, workers using this approach have often used a heterogeneous population of secreted molecules as the immunogen, and their only way of purifying the antibodies was affinity column chromatography. Although carefully controlled immunoprecipitations in the presence of excess unlabeled antigen were used in most of the studies, we believe that the identity of the antibody-precipitated protein should be documented by an exact, independent chemical method. In fact, immunologically positive results have been previously documented to be false when chemical characterization became available (7).

In this immunological approach, some are adding peptide mapping of the biosynthetic material. Under the best circumstances, peptide identification that depends solely on immunoprecipitation experiments followed by mapping of the protein fragments (especially by a one-dimensional technique) is at best indicative and preliminary.

We have recently investigated why the immunological approach should be limited and not be utilized to define biosynthetic models. While

looking at the different molecular forms of ACTH in human pituitary extract, we aimed at purifying some of them in sufficient quantity to characterize them chemically. One of these fractions, having an apparent molecular weight of 12,000 daltons, was purified using a highly specific ACTH radioimmunoassay. In all systems including molecular sieving on Biogel P30, HPLC reverse-phase column, HPLC molecular sieving column and SDS gel elctrophoresis, the material was eluted as a large molecular weight form of immunoreactive ACTH. The final product was highly pure and was analysed. The amino acid composition fitted exactly that of $ACTH^{1-39}$ (Table 1) and the sequencing revealed that, indeed, it was only 39 residues long (Figure 1).

Yet all the other results were compatible with a much larger molecular weight form. Although we have no explanation for the fact that this ACTH behave as a dimer or trimer, we are tempted to believe that these ACTH molecules have such a high chemical affinity that they cannot be separated under even the most drastic dissociating conditions.

We are thus convinced that many published results, lacking similar types of chemical data, have a certain degree of false positivity which explains the confusion which had been entertained until the sequence data became available.

TABLE 1
Amino Acid Composition of the Apparent 12K ACTH

Amino Acid	Residues per Molecule	
Asx	2.0	(2)
Ser	2.6	(3)
Glx	5.4	(5)
Gly	3.0	(3)
Ala	2.9	(3)
Val	2.9	(3)
Met	1.0	(1)
Leu	1.1	(1)
Tyr	1.9	(2)
Phe	3.0	(3)
His	1.0	(1)
Trp		(1)
Lys	3.7	(4)
Arg	3.0	(3)
Pro	4.7	(4)

Amino acid analysis (using 10–40 μg of peptide) was performed on the peptide following 22 h hydrolysis at 105°C in 5.7 N HCl in the presence of 0.1% β-mercaptoethanol. The separation of the amino acids was done on a modified 120 C Beckman amino acid analyzer using a Beckman W3 resin and single column methodology. Values are the average of two determinations. Numbers in parentheses refer to the amino acid composition of human ACTH consisting of the 39 residues.

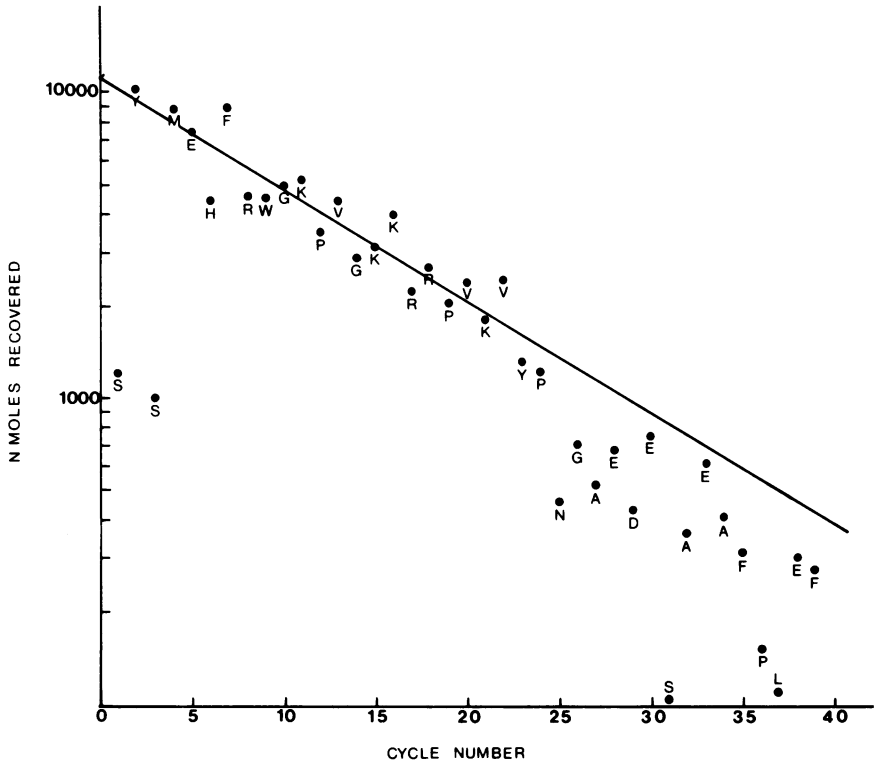

FIG. 1. Automatic NH2-terminal degradation of the apparent 12K ACTH. Sequencing was carried out on ≈10–15 nm of protein using 3 mg of polybrene precycled seven times and a 0.3 M Quadrol program on an updated Beckman 890B sequenator fitted with a cold trap. Phenylthiohydantoin (PTH) derivatives were obtained after conversion in methanol/HCl using an in-line sequemat P6 autoconverter. Separation and quantification were carried out by HPLC using an ultrasphere-ODS column and a tetrahydrofuran acetonitrile gradient. Quantitative yields of PTH-amino acids corrected for background and normalized to PTH-norleucine internal standard are illustrated as a function of residue number. The slope and intercept were obtained by a linear regression analysis on selected stable PTH-amino acids. Repetitive yield thus obtained was 91.9%.

2. PULSE AND PULSE-CHASE EXPERIMENTS

The β-LPH model was the first to be recognized by sequence analysis, while the pro-insulin model was first suggested following excellent pulse-chase experiments. Although pro-insulin was identified partly by some immunological characterization, the fact that one sees the dynamic change of a large precursor molecule to its final end-product gave good evidence of the precursor's existence (2). This approach was adopted for pro-PTH, the pro-glucagon model and by us for the POMC model (8–12).

3. Sequencing Approach

To us, this represents the definitive tool for validating biosynthetic models. Up to the middle 1970's, this was only possible through the amino acid sequencing of these molecules. In simpler models like LPH's, pro-insulin and pro-PTH, it was done appropriately and the results of pulse-chase experiments were confirmed. For the more complex models like POMC, enkephalin, dynorphin, calcitonin, somatostatin, vasopressin, etc, the development of DNA technology has brought tremendous advances in the field. As seen in Figure 2, the list of proven models is expanding rapidly and includes a great variety of peptide hormones, neuropeptides and growth factors.

In Figure 2, one can appreciate the fact that the presence of pairs of basic amino acids, first seen in 1967 in the β-LPH model, has become almost a universal rule. Moreover, as previously published (13, 14), the

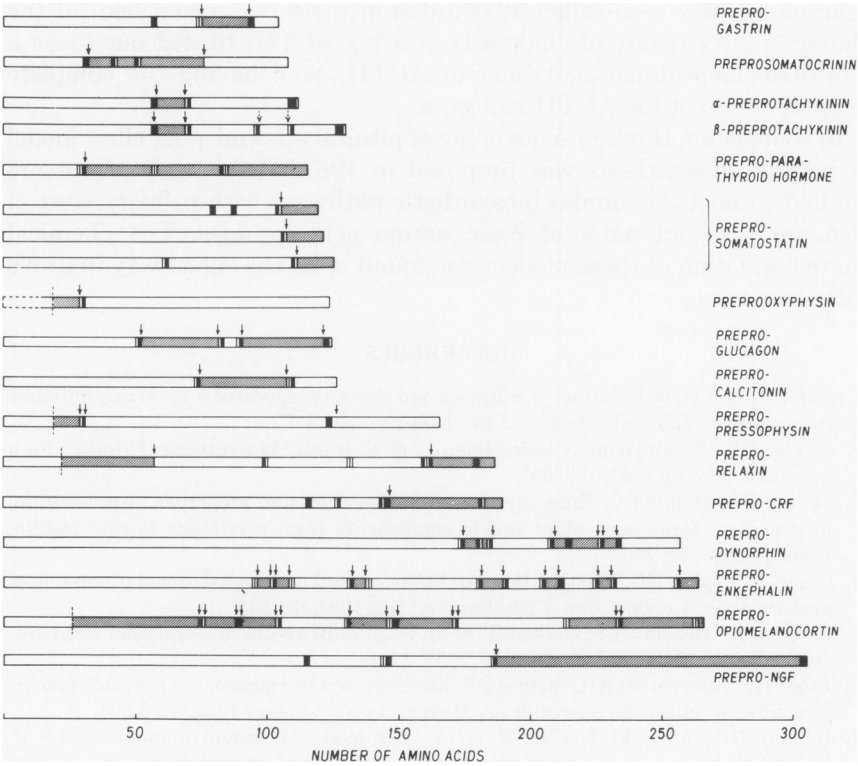

FIG. 2. Localization of basic pairs of amino acids inside the sequence of some precursor molecules. Those sequences were obtained by protein sequencing methods or cDNA-sequencing techniques. □ lysine; ■ arginine. Hatched areas correspond to peptides possessing known biological activities and obtained through cleavages shown by the arrows.

pair Lys-Arg, at least in the human pituitary gland, is the most frequent and is a preferential site of cleavage. It seems that other pairs of basic residues are less susceptible to proteolysis while single arginine residues are found sporadically. It is now also well recognized that a precursor like POMC will mature differently in different tissues. In a recent review, Krieger et al. (15) described this phenomenon extensively. POMC gives rise in the anterior lobe of the pituitary gland mainly to ACTH and β-LPH plus some endorphin, while the *pars intermedia* of the pituitary and the hypothalamus produces mainly α-MSH and β-endorphin. This interesting phenomenon is an indication of the variety of hormonal and neural peptides each precursor can produce.

In summary, the advent of protein microsequencing and DNA technology, is producing an exponential amount of new data in the field of peptide biosynthesis. These approaches are far superior to immunological characterization which, I suggest, should not be used for describing the chemical arrangements of the precursor models. We have shown an example of how a so-called 12,000 dalton molecule, which had all the characteristics (short of chemical) of a big ACTH, turned out to be a non-dissociable dimer or trimer of ACTH, each having the complete sequence 1–39 of the ACTH molecule.

In conclusion, through sequencing of pituitary β- and γ-LPH's a model of peptide biosynthesis was proposed in 1967. Most other precursors studied so far have similar biosynthetic pathways with primary sites of cleavage made of pairs of basic amino acid residues. The chemical characterization of these models was found to be the surest way to prove their existence.

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